Toxicon 58 (2011) 502–508

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Towards a better understanding of *Ipomoea asarifolia* toxicity: Evidence of the involvement of a leaf lectin

H.O. Salles [a], I.M. Vasconcelos [b], L.F.L. Santos [c], H.D. Oliveira [b], P.P.C. Costa [c], N.R.F. Nascimento [c], C.F. Santos [c], D.F. Sousa [d], A.R.C. Jorge [d], D.B. Menezes [e], H.S.A. Monteiro [d], D.M.F. Gondim [b], J.T.A. Oliveira [b,*]

[a] *Embrapa Caprinos e Ovinos, PO Box 145, 62010-970, Sobral, CE, Brazil*
[b] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Campus do Pici, 60451-970, Fortaleza, CE, Brazil*
[c] *Universidade Estadual do Ceará, Campus do Itaperi, 60740-000, Fortaleza, CE, Brazil*
[d] *Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, 60430-270, Fortaleza, CE, Brazil*
[e] *Departamento de Patologia e Medicina Legal, Universidade Federal do Ceará, 60441–750, Fortaleza, CE, Brazil*

ARTICLE INFO

Article history:
Received 15 April 2011
Received in revised form 22 July 2011
Accepted 18 August 2011
Available online 26 August 2011

Keywords:
Ipomoea asarifolia
Toxicity
Lectin

ABSTRACT

Natural intoxication of livestock by ingestion of *Ipomoea asarifolia* leaves has been reported to occur widely in Brazil. Previous studies carried out by our research group provided strong evidence that a lectin could be involved with the toxic properties of *I. asarifolia*. To reinforce this hypothesis, a lectin-enriched fraction (LEF) was isolated from *I. asarifolia* leaves and its toxic effects were assessed. Leaves of *I. asarifolia* were excised from plants growing widely in the field, mechanically wounded and maintained in a chamber at 25 ± 3 °C for 72 h in the dark, under near 100% relative humidity. The leaf proteins were extracted, ammonium sulfate precipitated, chromatographed on DEAE-cellulose and Phenyl-Sepharose to produce LEF that under SDS–PAGE showed a molecular mass of 44.0 kDa and after N-terminal amino acid analysis a primary sequence composed of AGYTPVLDIGAEVLAAGEPY. The *in vivo* toxicity of LEF assessed by intraorbital injection in mice showed induced severe uncoordinated movements without death. LEF reduced the muscular contraction in a dose depend way and at 29.8 μg/mL (CE_{50}) it produces 50% inhibition of contraction, suggesting that LEF blunts autonomic neurotransmission. Isolated rat kidneys were perfused with LEF and no effects on the perfusion pressure or renal vascular resistance were observed, but urinary flow and glomerular filtration rate increased. Moreover, the percentage of tubular transport of Na^+, K^+ and Cl^- decreased. Histological examination of the kidneys perfused with LEF exhibited little alterations. These toxic effects observed above were concomitant with the increase of LEF hemagglutination activity, which strongly suggest that one of the toxic principles of *I. asarifolia* is a lectin present in its leaves.

1. Introduction

Ipomoea asarifolia (Desr.) Roem. & Schult (common name: salsa or ginger-leaf morning-glory) is a tropical shrubby and quickly growing toxic plant of the Convolvulaceae family. Natural intoxication of livestock with *I. asarifolia* has been reported to occur widely in Brazil (Barbosa et al., 2005), particularly in Northeastern. Usually intoxication occurs when the animals feed on leaves as an alternative source of nutrients during dry seasons owing to food shortage (Medeiros et al., 2003). This intoxication is clinically characterized by mild depression, sleepiness, weak tremors of the head and neck muscles or discrete head nodding after exercise, severe lack of movement coordination, sideway

* Corresponding author. Tel.: +55 85 3366 9823; fax: +55 85 3366 9789.
E-mail address: jtaolive@ufc.br (J.T.A. Oliveira).

0041-0101 © 2011 Elsevier Ltd. Open access under the Elsevier OA license.
doi:10.1016/j.toxicon.2011.08.011

progression and fall, hypermetria, sway while standing and wide based stance (Medeiros et al., 2003).

Previously, it was suggested that the symptoms observed upon *I. asarifolia* consumption were due to lysosomal storage disease (Medeiros et al., 2000) as demonstrated for *Ipomoea sericophylla* and *Ipomoea riedelii* (Barbosa et al., 2006). However, no evidence of such disease was found after histological or ultrastructural evaluation of tissues or organs from goats experimentally intoxicated with *I. asarifolia* (Medeiros et al., 2003). In addition, the presence of negligible amount of swainsonine and the absence of calystegines in the samples of *I. asarifolia* used in previous experiments further suggest that the experimental intoxication induced by *I. asarifolia* in goats was probably not due to a storage disease (Medeiros et al., 2003). Actually, there are few studies on *I. asarifolia* toxicity and the toxic substances involved are unknown, and their mechanisms of action are not yet understood. Nevertheless, experimental evidence strongly suggested that a lectin present in the leaves of *I. asarifolia* could be involved in its toxic effects to goats (Santos, 2001).

Lectins are widely distributed in nature and several hundred of these molecules have been isolated from plants, viruses, bacteria, invertebrates and vertebrates, including mammals (Kennedy et al., 1995). Lectins are a class of proteins of non-immune origin, which possess at least one non-catalytic domain that specifically and reversibly bind to mono- or oligosaccharides (Peumans and Van Damme, 1995). A typical lectin has two or more carbohydrate-binding sites, being able to agglutinate cells. Thus they are commonly designated as agglutinins or hemagglutinins. Based on differences in molecular structures, biochemical properties, and carbohydrate-binding specificities, plant lectins are usually considered a complex and heterogeneous group of proteins with different pharmacological and toxicological properties.

This study was conducted to isolate a lectin-enriched fraction (LEF) from the leaves of *I. asarifolia* and assess its toxic effects on various models of study as an attempt to establish an association between this leaf lectin with the plant toxicity.

2. Materials and methods

2.1. Plant material and chemicals

I. asarifolia leaves were collected from naturally growing plants at the campus of Federal University of Ceará (UFC), Fortaleza, Brazil. A voucher specimen (registration number 040477) was deposited at Prisco Bezerra Herbarium of UFC, where it was botanically identified. The leaves were collected, washed with tap water to remove dust and mechanically wounded (four circular cuttings of 6 mm diameters equally distributed in the leaf blade) and maintained in a chamber at 25 ± 3 °C for 72 h, in the dark, under near 100% relative humidity. In a previous experiment it was noticed a significant increase of the hemagglutination activity upon leaf injury (data will be published elsewhere). After this, the leaves were powdered in the presence of liquid nitrogen and stored at −80 °C until required. DEAE-cellulose column was obtained from Whatman International Ltd., Maidstone, England; Phenyl-Sepharose 6-Fast Flow column was obtained from GE Healthcare, Uppsala, Sweden. Morphine was purchased from Sigma Aldrich Chemical (Saint Louis, MO, USA). The other chemicals were all of analytical grade and obtained from local suppliers.

2.2. Protein extraction and preparation of the lectin-enriched fraction (LEF)

The soluble proteins were extracted from the leaf powder with three volumes of 25 mM Tris–HCl, pH 7.5, supplemented with 3% (w/v) polyvinylpolypyrrolidone (PVPP) and 5 mM ascorbic acid, for 2 h at 4 °C, under gentle shaking. After filtration through nylon cloth, the filtrate was centrifuged at $10{,}000 \times g$ for 30 min, at 4 °C, and the supernatant (crude extract) recovered. The crude extract was precipitated with ammonium sulfate at 30% saturation (176 g/L) and the suspension maintained at 4 °C for 12 h. The precipitate obtained (Fraction 0–30%, shortly F030) after centrifugation ($10{,}000 \times g$, 40 min, 4 °C) was dialyzed exhaustively against Milli-Q grade water, lyophilized, and suspended in 25 mM Tris–HCl, pH 7.5. After centrifugation ($10{,}000 \times g$, 20 min, 4 °C), the fraction F030 was submitted to ion-exchange chromatography on a DEAE-cellulose column equilibrated with 25 mM Tris–HCl, pH 7.5. The through fraction was eluted from the column with the equilibrating buffer. The retained material was eluted with 25 mM Tris–HCl, pH 7.5, containing 200 mM NaCl, at a flow rate of 1 mL/min, dialyzed exhaustively against water and lyophilized. Next, it was suspended in 25 mM Tris–HCl, pH 7.5, containing 420 mM of ammonium sulfate, centrifuged ($10{,}000 \times g$, 20 min, 4 °C), and the supernatant obtained chromatographed on a Phenyl-Sepharose 6-Fast Flow column, equilibrated with the above buffer. The protein fraction obtained after elution with 25 mM Tris-HCl, pH 7.5, containing 100 mM of ammonium sulfate, at a flow rate of 1 mL/min, was dialyzed against Milli-Q grade water and lyophilized. This material represented the lectin-enriched fraction (LEF) that was characterized and used to assess toxicity.

2.3. Protein content

It was determined as previously described (Bradford, 1976). Absorbance at 280 nm was also used to monitor protein elution profiles during chromatographies.

2.4. Polyacrylamide gel electrophoresis

Protein fractions were analyzed by polyacrylamide gel electrophoresis (15% running gel, 3.5% stacking gel) (Laemmli, 1970). The samples were solubilized in 125 mM Tris–HCl buffer, pH 6.8, containing 2.6% (w/v) SDS, 0.5 mM EGTA, 0.5 mM EDTA, 12.6% (w/v) glycerol. Gels were stained with silver (Blum et al., 1986). Phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa) were used as molecular mass standards.

2.5. N-terminal amino acid sequence analysis

Following polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS–PAGE), LEF was transferred to polyvinylidene difluoride (PVDF) Hybond-P membrane (Amersham Biosciences) following the protocol described by Rybicki and Purves (1996) and stained with coomassie brilliant blue R-250. The protein band corresponding to LEF (44 kDa) was excised from the membrane and analyzed by automated Edman degradation, using a Shimadzu PPSQ-21/23 automated protein sequencer (Shimadzu, Kyoto, Japan). The amino acid sequence obtained was compared with other protein sequences deposited in the SWISS-PROT/TREMBL databases using the FASTA 3 and BLAST programs.

2.6. Hemagglutination assay

Hemagglutination activity was measured by a serial dilution procedure using a 2% suspension of trypsin-treated rabbit erythrocytes as previously described (Carbonaro et al., 2000) with some modifications. The assay was done in polystyrene microtiter U-bottomed 96-well plates and agglutination was visualized after 12 h. One hemagglutination unit (1 HU) was taken as the highest dilution giving complete agglutination of trypsin-treated rabbit erythrocytes.

2.7. Hemagglutination inhibition assay

Before the hemagglutination assay, two-fold serially diluted carbohydrate or glycoprotein samples (25 μL) in 150 mM NaCl were incubated for 30 min at 25 °C with 25 μL of LEF dissolved in 25 mM Tris–HCl, pH 7.5. The minimal concentration of carbohydrate or glycoprotein in the final reaction mixture capable of completely inhibiting 4 HU was recorded.

2.8. Stability of the hemagglutination activity

2.8.1. Effect of temperature

LEF solutions containing 0.0124 mg protein/mL in 25 mM Tris–HCl, pH 7.5, were heated at 70, 80, and 90 °C, from 5 to 60 min, at 5 min intervals. After cooling to 25 °C, the residual hemagglutination activity was assayed.

2.8.2. Effect of dithiotreitol (DTT)

LEF solutions containing 0.0124 mg protein/mL in 25 mM Tris–HCl, pH 7.5, were incubated for 60 min at 25 °C, in the presence of the reducing agent DTT at final concentrations of 5, 10, 50 and 100 mM and the residual hemagglutination activity measured.

2.8.3. Effect of protease treatments

LEF (1 mg) was incubated with 500 μL of pepsin (0.02 mg/mL of 100 mM HCl, pH 1.8) at 37 °C. After 2 h incubation, two 250 μL aliquots were withdrawal from the reaction mixture and 250 μL of 250 mM Tris–HCl, pH 8.9, were added to adjust pH to 8.0. Then 250 μL of a trypsin + chymotrypsin solution (0.02 mg/mL for each enzyme in 250 mM Tris-HCl, pH 8.9) were added to one of the pepsin hydrolysate (250 μL) and incubated for further 3 h, at 37 °C. The hemagglutinantion activity was analyzed for the hydrolyzates of LEF obtained after pepsin and pepsin followed by trypsin + chymotrypsin treatments.

2.9. NMR analysis

LEF (5 mg) was dissolved in 0.2 μL of 25 mM Tris–HCl, pH 7.5, containing 0.4 μL of D_2O. The NMR data were recorded using a Bruker Avance DPX300 spectrometer operating in the frequency of ^1H, at 300 MHz, to detect possible contamination by toxic secondary metabolite (swainsonine and calystegines, for example). The acquisition was made with 256 transients and 5 s relaxation time.

2.10. Biological assays

2.10.1. Intraorbital injection

LEF (5 mg/kg body weight) dissolved in 150 mM NaCl was injected intraorbitally in male Swiss mice (15.5–20.5 g body weight) to assess the toxicity *in vivo*. The animal behavior was observed for 1 h.

2.10.2. Mouse vas deferens assay

The electrically driven mouse vas deferens bioassay was performed as described by Henderson et al. (1972), using Swiss mice (38–42 g body weight). Vasa deferentia were inserted into silver ring electrodes, transferred to organ baths (5 mL capacity) set at 37 °C, and attached to force displacement transducers (F-60 Narco Biosystems, Houston, TX, USA) under a loading tension of 300 mg (2.94×10^{-3} N) to record motor responses isometrically. Concentration–response curves were obtained by cumulative addition of the crude extract to the bath medium at 2.5, 7.5, 25.0, 75.0, 250.0 and 750.0 μg protein/mL or LEF at 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, 100.0 and 300.0 μg protein/mL, both dissolved in Krebs solution. Stimulation of intramural nerves was carried out at a frequency of 0.1 Hz and duration of 10^{-3} s at supramaximal voltage (26 V). The motor responses of each cumulative dose were registered for 10 min. After the last dose, the system was washed three times with Krebs solution to remove the protein sample tested. Then, morphine (10 μM) was added to the organ bath to revert contractions elicited by electrical field stimulation as evidence that they were mainly of neurogenic origin.

2.10.3. Kidney perfusion

Adult Wistar rats (240–280 g body weight) were fasted with free access to water for 24 h before the experiments. The animals were anaesthetized with sodium pentobarbital (50 mg/kg body weight). The right renal artery was cannulated through the upper mesenteric artery, the kidney isolated and uninterrupted perfused with modified Krebs–Henseleit solution (MKHS), pH 7.4, at 37 °C, consisting (in mM) of: Na^+ 147.0; K^+ 5.0; Ca^{2+} 2.5; Mg^{2+} 2.0; Cl^- 110.0; HCO_3^- 2.5; SO_4^{2-} 1.0; PO_4^{3-} 1.0. This perfusion system was assembled according to Bowman (1970) and Fonteles et al. (1998). Bovine serum albumin (6% w/v, BSA fraction V, Sigma) was added to the modified MKHS and this solution was dialyzed for 48 h, at 4 °C, to remove citrate, piruvate and

lactate (Hanson and Ballard, 1968; Pegg, 1971). Next, 0.075 g urea, 0.075 g inulin and 0.15 g glucose were added and the pH adjusted to 7.4. This solution was gassed with a mixture of 95% O_2/5% CO_2 and the temperature stabilized at 37 °C. Perfusion pressure was determined at the tip of the stainless steel cannulae with a mercury manometer. Perfusate and urine samples were collected for Na^+, K^+, inulin and osmolarity determination. Na^+ and K^+ concentrations were determined by flame photometry (flame photometer Model 445; Micronal, Brazil), Cl^- using a kit (LABTEST, São Paulo, Brazil) and inulin according to Walser et al. (1955). Sample osmolality was measured using a WESCOR 5100c vapor pressure osmometer (WESCOR, Needham Heights, MA, USA). Glomerular filtration rate (GFR), fractional tubular transport of sodium (%TNa^+), potassium (%TK^+) and chloride (%TCl^-), urinary flow (UF) and renal vascular resistance (RVR) were determined as previously described (Walser et al., 1955). The data were evaluated using analysis of variance followed by Student's *t*-test. Results are expressed as mean ± SEM with the level of significance set at 5% ($P < 0.05$; $n = 4$). After the experiment, both right and left kidneys were removed and fixed in 10% formaldehyde for histological processing and examination. The experiments followed the methodology recommended by International Ethical Standards in animal research and was approved by the Scientific and Ethical Committee of the Federal University of Ceará, Brazil.

3. Results

3.1. Isolation and characterization of LEF

The crude extract of *I. asarifolia* leaves injured and kept in the dark for 72 h gave a hemagglutination specific activity of 650.9 ± 16.7 HU/mg protein whereas the crude extract from uninjuried leaves not kept in the dark (control) gave 416.9 ± 2.7 HU/mg protein. The increase in activity in wounded\darkened leaves was due to the *de novo* synthesis of the native leaf lectin because irrespective of wound treatment the lectin-enriched fraction (LEF) gave the same N-terminal amino acid sequence. Thus the starting material for LEF production was the crude extract of wounded/darkened leaves.

This lectin-enriched fraction (LEF) from *I. asarifolia* leaves was obtained after protein extraction, ammonium sulfate precipitation, DEAE-cellulose and Phenyl-Sepharose 6-Fast Flow chromatographies, as detailed in 2.2. SDS–PAGE of LEF showed a main protein band with relative molecular weight of around 44.0 kDa (Fig. 1, Lane 3). This band was electroblotted onto PVDF membrane and had its N-terminal amino acid sequence determined: AVNLPAGHLSP GGVGNYVVTVGLCTP.

LEF had a specific hemagglutination activity of 1118 HU/mg protein. It was inhibited by the glycoprotein fetuin (3.0×10^{-3} mM), as well as by sialic acid (N-acetyl-D-neuramic acid, minimum inhibitory concentration of 3.0 mM) (Santos, 2001) which is a component of fetuin. However it was not inhibited by the simple sugars D-arabinose, L-fructose, D-galactose, N-acetyl-D-galactosamine, D-glucose, N-acetyl-D-glucosamine, D-mannose, D-xilose, the disaccharides α-lactose, maltose, sacarose, and the trisaccharide

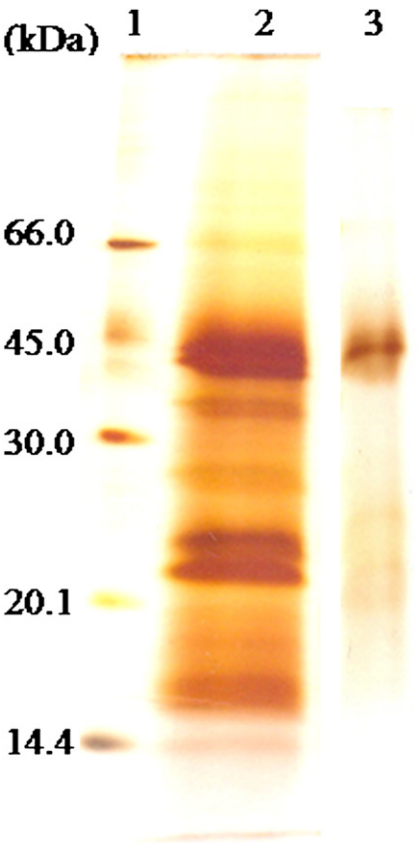

Fig. 1. SDS–PAGE (15% polyacrylamide) of the protein fractions (3 μg) extracted from *I. asarifolia* leaves. Lane 1, Molecular mass markers; Lane 2, fraction eluted from DEAE-cellulose column with 200 mM NaCl; Lane 3, LEF preparation eluted from phenyl-sepharose column with 100 mM ammonium sulfate.

D-raffinose, even at high concentration (100 mM), neither by the glycoproteins BSA and mucin (Santos, 2001).

Heat treatment at 70 and 80 °C for 60 min reduced LEF agglutination activity against trypsin-treated rabbit erythrocytes to 75% and at 90 °C it was completely abolished within 10 min (Fig. 2). Treatment of LEF with DTT (5, 10, 50

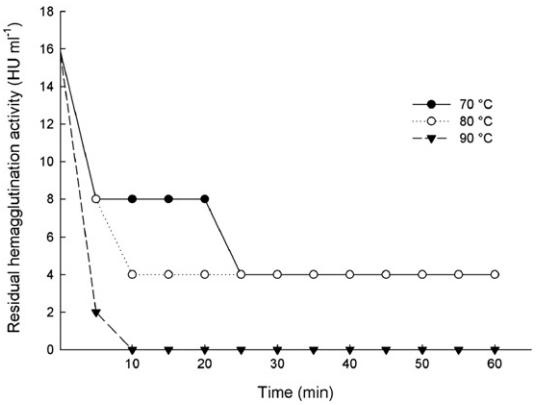

Fig. 2. Thermal stability of LEF. Protein concentration was 0.0124 mg/ml of 25 mM Tris–HCl buffer, pH 7.5.

or 100 mM) had no influence on the hemagglutination activity. *In vitro* digestion of LEF with pepsin alone or pepsin followed by trypsin and chymotrypsin did not inactivate its hemagglutination activity.

3.2. Biological assays

The biological assays done in this study were conducted with the LEF preparation showed in Fig. 1, lane 3. This preparation was free of secondary compounds as evaluated by NMR analysis (data not shown).

The *in vivo* toxicity of LEF assessed by intraorbital injection in mice showed induced severe uncoordinated movements without death. Regarding to vas deferens stimulation, the crude extract and LEF from *I. asarifolia* leaves reduced the muscular contraction in a dose depend way (Fig. 3). The concentrations able to produce 50% inhibition of contraction (CE_{50}) were 52.2 μg/mL and 29.8 μg/mL for the crude extract and LEF, respectively, showing that LEF was more effective than the crude extract. Nevertheless, these findings suggest that both protein preparations blunt autonomic neurotransmission. The neurogenic contractions were completely recovered after withdrawal of LEF through three washings of the system. One plausible hypothesis that could be put forward in relation to the contraction recovery after removal of LEF by washing is that the binding of the lectin to receptors is weak. Nevertheless, most important is that the presence of LEF is essential for the elicitation of the effects observed.

There are some published data that show anatomopathologic alterations in the kidneys of experimental animals fed on *I. asarifolia* leaves such as nephron destruction/degeneration and necrosis of the epithelial cells of the renal cortex and renal medulla of mice and sheep (Santos, 2001; Chaves, 2009). In our study isolated kidneys perfused with LEF (10 μg/mL) had no effect on the perfusion pressure or renal vascular resistance. Contrary, urinary flow and glomerular filtration rate started to increase at 60 min (Fig. 4A and B). The percentage of the tubular transport of

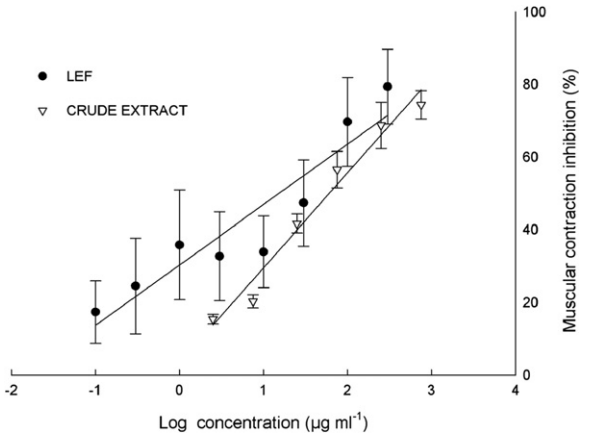

Fig. 3. Effects of cumulative doses of crude extract and LEF from *I. asarifolia* leaves on the neurogenic contractions of mouse vas deferens muscle. Crude extract concentrations were 2.5; 7.5; 25.0; 75.0; 250.0; 750.0 μg protein/ml. LEF concentrations were 0.1; 0.3; 1.0; 3.0; 10.0; 30.0; 100.0; 300.0 μg protein/ml.

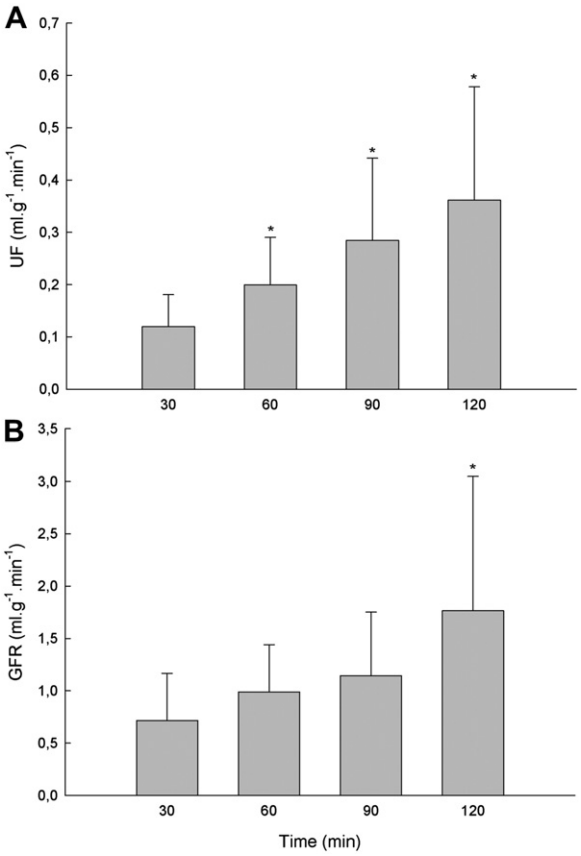

Fig. 4. The effect of LEF (10 μg protein/ml) on renal urinary flow (A) and glomerular filtration rate (B). The first 30 min of perfusion with supplemented MKHS without LEF was taken as control. The data are reported as mean ± SEM ($n = 4$ rats). *Significant ($P < 0.05$) compared with control group.

sodium (%TNa^+), potassium (%TK^+), and chloride (%Cl^-) decreased at 90 min (Fig. 5) as compared with control (kidneys perfused for 30 min with supplemented MKHS without LEF). Histological examination of the kidneys that received the perfusion treatment with LEF exhibited little alterations, but deposits of proteinaceous material in the tubules and/or glomerules were observed for some specimens in comparison with controls that were not exposed to LEF. No abnormalities were observed in renal vessels or urinary space.

4. Discussion

Ipomoea species grow naturally or are cultivated in various regions of the world because of their ornamental bright colored flowers. However, it is well known that some *Ipomoea* species are very toxic (Medeiros et al., 2003; Barbosa et al., 2006). In Northeastern Brazil wildly growing *Ipomoea asarifolia* causes natural intoxication in goat, sheep and bovine (Barbosa et al., 2005) particularly during drought periods when food is scarce. Experimentally, animals such as buffaloes (Barbosa et al., 2005) and mouse (Santos, 2001), which are not naturally intoxicated by *Ipomoea* species, have

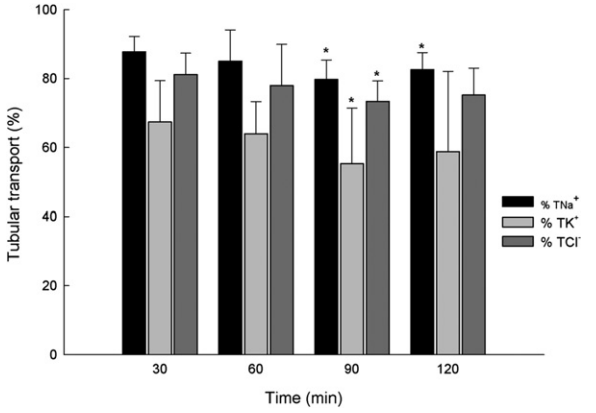

Fig. 5. The effect of LEF (10 μg protein/ml) on the percentage of sodium (% TNa^+), potassium (%TK^+) and chloride (%TCl^-) tubular transport. The first 30 min of perfusion with supplemented MKHS without LEF was taken as control. Data are reported as mean ± SEM ($n = 4$ rats). *Significant ($P < 0.05$) compared with control group.

been used to study and understand their toxic effects (Hueza et al., 2005).

Previous studies carried out by our research group showed that the amount of LEF found in *I. asarifolia* is around 1.0 mg/100 g dry leaves and provided evidence that this lectin could be involved in the toxic properties of *I. asarifolia* (Santos, 2001). To test this hypothesis, we isolated a lectin-enriched fraction (LEF) from *I. asarifolia* leaves composed of a 44.0 kDa protein band (Fig. 1, lane 3) that presented high hemagglutination activity against trypsin-treated rabbit erythrocytes. The N-terminal sequence of LEF has 69%, 65%, 65% and 38% identity with the 41 kDa chloroplast nucleoid DNA-binding proteins (CND-41) of *Oryza sativa* subsp. *Japonica, Nicotiana tabacum*, *Nicotiana sylvestris* and *Arabidopsis thaliana,* respectively (Murakami et al., 2000; Nakano et al. 1993, 1997). Nakano et al. (1997) observed that CND-41 was rare in actively photosynthesizing cells and/or tissues and suggested that CND-41 acts as a negative regulator of chloroplast gene expression. Incidentally, in this study, the leaves of *I. asarifolia* were kept in the dark after mechanically wounded.

Our research group showed that LEF has affinity for fetuin, a glycoprotein that has sialic acid at the terminal sugar residues (Ashida et al., 2000) and for N-acetyl-D-neuramic acid (sialic acid) (Santos, 2001 and this study). Sialic acid is a component of the cell plasma membrane that modulates signal transduction particularly in gangliosides, a class of complex glycosphingolipids present in neuronal cell membranes (Mlinac and Bognar, 2010). There is evidence that sialic acids mediate specific cellular and molecular recognition by regulating association with glycan-binding proteins such as lectins (Zhuo and Bellis, 2011). Therefore, there are potential bind sites for LEF in animal cells, especially in the neural tissue. Of particular interest is the finding that the hemagglutination activity of LEF was not abolished by the *in vitro* digestion with the proteolytic enzymes pepsin, trypsin and chymotrypsin. Several plant lectins are known to survive *in vivo* the breakdown by proteolytic enzymes and interact with cell surface sugar receptors, mediating endocytosis, an essential event that precedes cellular toxicity (Vasconcelos and Oliveira, 2004). Thus it is possible that, *in vivo*, LEF binds to sialic acid bearing receptors in the goat gut cells allowing its systemic internalization and disturbance of the neural system. For instance, Ríos et al. (2008) carried out a histopathologic study that revealed the presence of cytoplasmatic vacuolation mainly in medulla oblongata and cerebellum of 1–3-year-old goats that received daily oral doses of 50 g/kg body weight of fresh leaves, flowers and stems of *Ipomoea carnea*, during 43–60 days. *I. carnea* is also a poisonous plant to cattle, sheep and goats (Tokarnia et al., 2002). The effect of *I. asarifolia* upon autonomic neurotransmission has never been assessed before. In this study, inhibition of autonomic neurotransmission of mouse vas deferens by LEF indicated that this fraction has neurotoxic properties. Indeed, LEF was more effective than the leaf crude extract regarding to inhibition of autonomic neurotransmission in mouse.

In regard to the renal effects caused by exposure to LEF, no changes on perfusion pressure and renal vascular resistance were observed. These data suggest that LEF did not induce vascular effect as observed by exposure to the lectins from *Canavalia brasiliensis* (ConBr), *Canavalia ensiformis* (ConA), *Dioclea guianensis* (DguiL) and *Vatairea macrocarpa* (Teixeira et al., 2001; Havt et al., 2003; Martins et al., 2005). As LEF has different carbohydrate specificity compared to ConBr, ConA, DguiL and *Vatairea macrocarpa* lectin, it might not have interacted with the target site that triggers changes on perfusion pressure and renal vascular resistance.

The increase in glomerular filtration rate (Fig. 4) and decrease in the percentage of $Na^+/K^+/Cl^-$ tubular transport (Fig. 5), both induced by LEF-perfusion, produced a tubuloglomerular feedback alteration which is a complex process that regulates the glomerular filtration rate. Interference in $Na^+/K^+/Cl^-$ transport and increase in glomerular filtration rate was also observed in ConBr-perfused rat kidney (Teixeira et al., 2001). However, ConA affected only K^+ reabsorption (Havt et al., 2003) and *V. macrocarpa* lectin had no interference with electrolyte transport, but increased the glomerular filtration rate and the urinary flow (Martins et al., 2005). Nevertheless, these above lectin-associated effects suggest the possible involvement of carbohydrate specific target receptors on the animal cell recognized by lectins.

In conclusion, the toxic effects observed in the various models used in this study when exposed to LEF strongly suggest that one of the toxic principles of *I. asarifolia* is a sialic acid binding lectin present in its leaves.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We thank EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) for partially support this research and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico (GS1)) for doctoral scholarship (Grant no. 081408/2003-05) to H.O. Salles. We also thank to Centro Nordestino de Aplicação e uso da Ressonância Magnética Nuclear (CENAUREMN) of Federal University of Ceará for NMR

analyze, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Programa Nacional de Cooperação Acadêmica (PROCAD) and Fundação de Amparo à Pesquisa do Estado do Ceará (FUNCAP).

References

Ashida, H., Yamamoto, K., Kumagai, H., 2000. Trypsin inhibitory activity of bovine fetuin de- *O*-glycosylated by endo-alpha- *N*-acetylgalactosaminidase. Biosci. Biotechnol. Biochem. 64, 2266–2268.

Barbosa, J.D., de Oliveira, C.M.C., Duarte, M.D., Peixoto, P.V., Tokarnia, C.H., 2005. Experimental and natural intoxication of buffaloes and other ruminants with *Ipomoea asarifolia* (Convolvulaceae). Pesq. Vet. Bras. 25, 231–234.

Barbosa, R.C., Riet-Correa, F., Medeiros, R.M.T., Lima, E.F., Barros, S.S., Gimeno, E.J., Molyneux, R.J., Gardner, D.R., 2006. Intoxication by *Ipomoea sericophylla* and *Ipomoea riedelii* in goats in the state of Paraíba, Northeastern Brazil. Toxicon 47, 371–379.

Blum, H., Beier, H., Gross, H.J., 1986. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99.

Bowman, R.H., 1970. Gluconeogenesis in the isolated perfused rat kidney. J. Biol. Chem. 245, 1604–1612.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72, 248–254.

Carbonaro, M., Grant, G., Cappelloni, M., Pusztai, A., 2000. Perspectives into factors limiting *in vivo* digestion of legume proteins: antinutritional compounds or storage proteins? J. Agric. Food Chem. 48, 742–749.

Chaves, D.P., 2009. Experimental intoxication by *Ipomoea asarifolia* in sheep: clinical, laboratorial and anatomopathologic findings. São Paulo State University, São Paulo, Brazil, doctoral thesis, 70 pp.

Fonteles, M.C., Greenberg, R.N., Monteiro, H.S.A., Currie, M.G., Forte, L.R., 1998. Natriuretic and kaliuretic activities of guanylin and uroguanylin in the isolated perfused rat kidney. Am. J. Phisiol. 275, 191–197.

Hanson, R.W., Ballard, F.S., 1968. Citrate, pyruvate and lactate contaminants of commercial serum albumin. J. Lipid Res. 9, 667–668.

Havt, A., Barbosa, P.S.F., Sousa, T.M., Martins, A.M.C., Nobre, A.C.L., Nascimento, K.S., Teixeira, E.H., Pinto, V.P.T., Sampaio, A.H., Fonteles, M.C., Cavada, B.S., Monteiro, H.S.A., 2003. Renal alterations promoted by the lectins from *Canavalia ensiformis* (Con A) and *Dioclea guianensis* (DguiL) seeds. Protein Pept. Lett. 10, 1–7.

Henderson, G., Hughes, J., Kosterlitz, H.W., 1972. A new example of a morphine-sensitive neuro-effector junction: adrenergic transmission in the mouse vas deferens. Br. J. Pharmacol. 46, 764–766.

Hueza, I.M., Guerra, J.L., Haraguchi, M., Asano, N., Górniak, S.L., 2005. The role of alkaloids in *Ipomoea carnea* toxicosis: a study in rats. Exp. Toxicol. Pathol. 57, 53–58.

Kennedy, J.F., Palva, P.M.G., Corella, M.T.S., Cavalcanti, M.S.M., Coelho, L.C. B.B., 1995. Lectins, versatile proteins of recognition: a review. Carbohydr. Polym. 26, 19–230.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227, 680–685.

Martins, A.M.C., Monteiro, A.M.O., Havt, A., Barbosa, P.S.F., Soares, T.F., Evangelista, J.S.A.M., de Menezes, D.B., Fonteles, M.C., Teixeira, E.H., Pinto, V.P.T., Nascimento, K.S., Alencar, N.M.N., Cavada, B.S., Monteiro, H.S.A., 2005. Renal effects induced by the lectin from *Vatairea macrocarpa* seeds. J. Pharm. Pharmacol. 57, 1329–1333.

Medeiros, R.M.T., Barbosa, R.C., Riet-Correa, F., Lima, E.F., Tabosa, I.M., de Barros, S.S., Gardner, D.R., Molyneux, R.J., 2003. Tremorgenic syndrome in goats caused by *Ipomoea asarifolia* in Northeastern Brazil. Toxicon 41, 933–935.

Medeiros, R.M.T., Nobre, V.M.T., Tabosa, I.M., Riet-Correa, F., 2000. Toxic plants for ruminants in the state of Paraíba, Northeastern Brazil. World Buiatrics Congress (CD Rom) 21, 10,141–10,150.

Mlinac, K., Bognar, S.K., 2010. Role of gangliosides in brain aging and neurodegeneration. Transl. Neurosci. 1, 300–307.

Murakami, S., Kondo, Y., Nakano, T., Sato, F.O.Y., 2000. Protease activity of CND41, a chloroplast nucleoid DNA-binding protein, isolated from cultured tobacco cells. FEBS Lett. 468, 15–18.

Nakano, T., Sato, F., Yamada, Y., 1993. Analysis of nucleoid-proteins in tobacco chloroplasts. Plant Cell Physiol. 34, 873–886.

Nakano, T., Murakami, S., Shoji, T., Yoshida, S., Yamada, Y., Sato, F., 1997. A nove1 protein with DNA binding activity from tobacco chloroplast nucleoids. Plant Cell 9, 1673–1682.

Pegg, D.E., 1971. Vascular resistance of the isolated rabbit kidney. Cryobiology 8, 431–440.

Peumans, W.J., Van Damme, J.M., 1995. Lectins as plant defense proteins. Plant Physiol. 109, 347–352.

Ríos, E., Cholich, L., Silva, J., Acosta de Pérez, O., 2008. Histopathological lesions in central nervous system of goats poisoned by *Ipomoea carnea*. Rev. Vet. 19, 130–134.

Rybicki, E.P., Purves, M., 1996. Western or enzyme-assisted immunoelectro-blotting (IEB). In: Coyne, V.E., James, M.D., Reid, S.J., Rybicki, E. P. (Eds.), Molecular Biology Techniques Manual, third ed. http://biowww.net/detail-311.html.

Santos, L.F.L., 2001. Salsa (*Ipomoea asarifolia* R. et Schult.) toxin: biochemical, structural, functional aspects and biotechnological potential. Federal Universisity of Ceará, Fortaleza, Ceará, Brazil, Doctoral Thesis; 142 pp.

Teixeira, E.H., Havt, A., Barbosa, P.S.F., Menezes, D.B., Fonteles, M.C., Monteiro, H.S.A., Sampaio, A.H., Cavada, B.S., 2001. Renal effects of the lectin from *Canavalia brasiliensis* seeds. Prot. Pep. Lett. 8, 477–484.

Tokarnia, C.H., Dobereiner, J., Peixoto, P.V., 2002. Poisonous plants affecting livestock in Brazil. Toxicon 40, 1635–1660.

Vasconcelos, I.M., Oliveira, J.T.A., 2004. Antinutritional properties of plant lectins. Toxicon 44, 385–403.

Walser, M., Davidson, D.G., Orloff, J., 1955. The renal clearance of alkali-stable inulin. J. Clin. Invest. 34, 1520–1523.

Zhuo, Y., Bellis, S.L., 2011. Emerging role of 2,6-sialic acid as a negative regulator of galectin binding and function. J. Biol Chem. 286, 5935–5941.